1   CANCEROUS DISEASE MODIFYING ANTIBODIES

2   <u>Field of the Invention:</u>

3   This invention relates to the isolation and production of cancerous disease

4   modifying antibodies (CDMAB) and to the use of these CDMAB in therapeutic and

5   diagnostic processes, optionally in combination with one or more chemotherapeutic agents.

6   The invention further relates to binding assays, which utilize the CDMAB of the instant

7   invention.

8   <u>Background of the Invention:</u>

9   Each individual who presents with cancer is unique and has a cancer that is as

10   different from other cancers as that person's identity. Despite this, current therapy treats

11   all patients with the same type of cancer, at the same stage, in the same way. At least 30

12   percent of these patients will fail the first line therapy, thus leading to further rounds of

13   treatment and the increased probability of treatment failure, metastases, and ultimately,

14   death. A superior approach to treatment would be the customization of therapy for the

15   particular individual. The only current therapy, which lends itself to customization, is

16   surgery. Chemotherapy and radiation treatment cannot be tailored to the patient, and

17   surgery by itself, in most cases is inadequate for producing cures.

18   With the advent of monoclonal antibodies, the possibility of developing methods

19   for customized therapy became more realistic since each antibody can be directed to a

20   single epitope. Furthermore, it is possible to produce a combination of antibodies that are

21   directed to the constellation of epitopes that uniquely define a particular individual's

22   tumor.

1   Having recognized that a significant difference between cancerous and normal cells

2   is that cancerous cells contain antigens that are specific to transformed cells, the scientific

3   community has long held that monoclonal antibodies can be designed to specifically target

4   transformed cells by binding specifically to these cancer antigens; thus giving rise to the

5   belief that monoclonal antibodies can serve as "Magic Bullets" to eliminate cancer cells.

6   Monoclonal antibodies isolated in accordance with the teachings of the instantly

7   disclosed invention have been shown to modify the cancerous disease process in a manner

8   which is beneficial to the patient, for example by reducing the tumor burden, and will

9   variously be referred to herein as cancerous disease modifying antibodies (CDMAB) or

10 "anti-cancer" antibodies.

11   At the present time, the cancer patient usually has few options of treatment. The

12 regimented approach to cancer therapy has produced improvements in global survival and

13 morbidity rates. However, to the particular individual, these improved statistics do not

14 necessarily correlate with an improvement in their personal situation.

15   Thus, if a methodology was put forth which enabled the practitioner to treat each

16 tumor independently of other patients in the same cohort, this would permit the unique

17 approach of tailoring therapy to just that one person. Such a course of therapy would,

18 ideally, increase the rate of cures, and produce better outcomes, thereby satisfying a long-

19 felt need.

20   Historically, the use of polyclonal antibodies has been used with limited success in

21 the treatment of human cancers. Lymphomas and leukemias have been treated with human

22 plasma, but there were few prolonged remissions or responses. Furthermore, there was a

1   lack of reproducibility and no additional benefit compared to chemotherapy. Solid tumors

2   such as breast cancers, melanomas and renal cell carcinomas have also been treated with

3   human blood, chimpanzee serum, human plasma and horse serum with correspondingly

4   unpredictable and ineffective results.

5   There have been many clinical trials of monoclonal antibodies for solid tumors. In

6   the 1980s there were at least 4 clinical trials for human breast cancer which produced only

7   1 responder from at least 47 patients using antibodies against specific antigens or based on

8   tissue selectivity. It was not until 1998 that there was a successful clinical trial using a

9   humanized anti-Her2 antibody in combination with Cisplatin. In this trial 37 patients were

10   accessed for responses of which about a quarter had a partial response rate and another half

11   had minor or stable disease progression.

12   The clinical trials investigating colorectal cancer involve antibodies against both

13   glycoprotein and glycolipid targets. Antibodies such as 17-1A, which has some specificity

14   for adenocarcinomas, had undergone Phase 2 clinical trials in over 60 patients with only 1

15   patient having a partial response. In other trials, use of 17-1A produced only 1 complete

16   response and 2 minor responses among 52 patients in protocols using additional

17   cyclophosphamide. Other trials involving 17-1A yielded results that were similar. The use

18   of a humanized murine monoclonal antibody initially approved for imaging also did not

19   produce tumor regression. To date there has not been an antibody that has been effective

20   for colorectal cancer. Likewise there have been equally poor results for lung cancer, brain

21   cancers, ovarian cancers, pancreatic cancer, prostate cancer, and stomach cancer. There

22   has been some limited success in the use of anti-GD3 monoclonal antibody for melanoma.

1   Thus, it can be seen that despite successful small animal studies that are a prerequisite for

2   human clinical trials, the antibodies that have been tested thus far have been, for the most

3   part, ineffective.

4   Prior Patents:

5   U.S. Patent No. 5,750,102 discloses a process wherein cells from a patient's tumor

6   are transfected with MHC genes, which may be cloned from cells or tissue from the

7   patient. These transfected cells are then used to vaccinate the patient.

8   U.S. Patent No. 4,861,581 discloses a process comprising the steps of obtaining

9   monoclonal antibodies that are specific to an internal cellular component of neoplastic and

10   normal cells of the mammal but not to external components, labeling the monoclonal

11   antibody, contacting the labeled antibody with tissue of a mammal that has received

12   therapy to kill neoplastic cells, and determining the effectiveness of therapy by measuring

13   the binding of the labeled antibody to the internal cellular component of the degenerating

14   neoplastic cells.   In preparing antibodies directed to human intracellular antigens, the

15   patentee recognizes that malignant cells represent a convenient source of such antigens.

16   U.S. Patent No. 5,171,665 provides a novel antibody and method for its production.

17   Specifically, the patent teaches formation of a monoclonal antibody which has the property

18   of binding strongly to a protein antigen associated with human tumors, e.g. those of the

19   colon and lung, while binding to normal cells to a much lesser degree.

20   U.S. Patent No. 5,484,596 provides a method of cancer therapy comprising

21   surgically removing tumor tissue from a human cancer patient, treating the tumor tissue to

22   obtain tumor cells, irradiating the tumor cells to be viable but non-tumorigenic, and using

1   these cells to prepare a vaccine for the patient capable of inhibiting recurrence of the

2   primary tumor while simultaneously inhibiting metastases.   The patent teaches the

3   development of monoclonal antibodies, which are reactive with surface antigens of tumor

4   cells. As set forth at col. 4, lines 45 et seq., the patentees utilize autochthonous tumor cells

5   in the development of monoclonal antibodies expressing active specific immunotherapy in

6   human neoplasia.

7   U.S. Patent No. 5,693,763 teaches a glycoprotein antigen characteristic of human

8   carcinomas is not dependent upon the epithelial tissue of origin.

9   U.S. Patent No. 5,783,186 is drawn to anti-Her2 antibodies, which induce apoptosis

10   in Her2 expressing cells, hybridoma cell lines producing the antibodies, methods of

11   treating cancer using the antibodies and pharmaceutical compositions including said

12   antibodies.

13   U.S. Patent No. 5,849,876 describes new hybridoma cell lines for the production of

14   monoclonal antibodies to mucin antigens purified from tumor and non-tumor tissue

15   sources.

16   U.S. Patent No. 5,869,268 is drawn to a method for generating a human

17   lymphocyte producing an antibody specific to a desired antigen, a method for producing a

18   monoclonal antibody, as well as monoclonal antibodies produced by the method. The

19   patent is particularly drawn to the production of an anti-HD human monoclonal antibody

20   useful for the diagnosis and treatment of cancers.

21   U.S. Patent No. 5,869,045 relates to antibodies, antibody fragments, antibody

22   conjugates and single chain immunotoxins reactive with human carcinoma cells. The

1 mechanism by which these antibodies function is two-fold, in that the molecules are

2 reactive with cell membrane antigens present on the surface of human carcinomas, and

3 further in that the antibodies have the ability to internalize within the carcinoma cells,

4 subsequent to binding, making them especially useful for forming antibody-drug and

5 antibody-toxin conjugates. In their unmodified form the antibodies also manifest cytotoxic

6 properties at specific concentrations.

7 U.S. Patent No. 5,780,033 discloses the use of autoantibodies for tumor therapy and

8 prophylaxis. However, this antibody is an anti-nuclear autoantibody from an aged

9 mammal. In this case, the autoantibody is said to be one type of natural antibody found in

10 the immune system. Because the autoantibody comes from "an aged mammal", there is no

11 requirement that the autoantibody actually comes from the patient being treated. In

12 addition the patent discloses natural and monoclonal anti-nuclear autoantibody from an

13 aged mammal, and a hybridoma cell line producing a monoclonal anti-nuclear

14 autoantibody.

15 Summary of the Invention:

16 The instant inventors have previously been awarded U.S. Patent 6,180,357, entitled

17 "Individualized Patient Specific Anti-Cancer Antibodies" directed to a process for

18 selecting individually customized anti-cancer antibodies, which are useful in treating a

19 cancerous disease.

20 This application utilizes, in part, the method for producing patient specific anti-

21 cancer antibodies as taught in the '357 patent for isolating hybridoma cell lines which

22 encode for cancerous disease modifying monoclonal antibodies. These antibodies can be

1   made specifically for one tumor and thus make possible the customization of cancer

2   therapy. Within the context of this application, anti-cancer antibodies having either cell

3   killing (cytotoxic) or cell-growth inhibiting (cytostatic) properties will hereafter be referred

4   to as cytotoxic. These antibodies can be used in aid of staging and diagnosis of a cancer,

5   and can be used to treat tumor metastases.

6   The prospect of individualized anti-cancer treatment will bring about a change in

7   the way a patient is managed. A likely clinical scenario is that a tumor sample is obtained

8   at the time of presentation, and banked. From this sample, the tumor can be typed from a

9   panel of pre-existing cancerous disease modifying antibodies. The patient will be

10 conventionally staged but the available antibodies can be of use in further staging the

11 patient. The patient can be treated immediately with the existing antibodies, and a panel of

12 antibodies specific to the tumor can be produced either using the methods outlined herein

13 or through the use of phage display libraries in conjunction with the screening methods

14 herein disclosed. All the antibodies generated will be added to the library of anti-cancer

15 antibodies since there is a possibility that other tumors can bear some of the same epitopes

16 as the one that is being treated. The antibodies produced according to this method may be

17 useful to treat cancerous disease in any number of patients who have cancers that bind to

18 these antibodies.

19   In addition to anti-cancer antibodies, the patient can elect to receive the currently

20 recommended therapies as part of a multi-modal regimen of treatment. The fact that the

21 antibodies isolated via the present methodology are relatively non-toxic to non-cancerous

22 cells allows for combinations of antibodies at high doses to be used, either alone, or in

1   conjunction with conventional therapy. The high therapeutic index will also permit re-

2   treatment on a short time scale that should decrease the likelihood of emergence of

3   treatment resistant cells.

4   Furthermore, it is within the purview of this invention to conjugate standard

5   chemotherapeutic modalities, e.g. radionuclides, with the CDMAB of the instant invention,

6   thereby focusing the use of said chemotherapeutics.

7   If the patient is refractory to the initial course of therapy or metastases develop, the

8   process of generating specific antibodies to the tumor can be repeated for re-treatment.

9   Furthermore, the anti-cancer antibodies can be conjugated to red blood cells obtained from

10   that patient and re-infused for treatment of metastases. There have been few effective

11   treatments for metastatic cancer and metastases usually portend a poor outcome resulting

12   in death. However, metastatic cancers are usually well vascularized and the delivery of

13   anti-cancer antibodies by red blood cells can have the effect of concentrating the antibodies

14   at the site of the tumor. Even prior to metastases, most cancer cells are dependent on the

15   host's blood supply for their survival and anti-cancer antibodies conjugated to red blood

16   cells can be effective against *in situ* tumors as well. Alternatively, the antibodies may be

17   conjugated to other hematogenous cells, e.g. lymphocytes, macrophages, monocytes,

18   natural killer cells, etc.

19   There are five classes of antibodies and each is associated with a function that is

20   conferred by its heavy chain. It is generally thought that cancer cell killing by naked

21   antibodies are mediated either through antibody dependent cellular cytotoxicity (ADCC) or

22   complement dependent cytotoxicity (CDC). For example murine IgM and IgG2a

1   antibodies can activate human complement by binding the C-1 component of the

2   complement system thereby activating the classical pathway of complement activation,

3   which can lead to tumor lysis. For human antibodies the most effective complement

4   activating antibodies are generally IgM and IgG1. Murine antibodies of the IgG2a and

5   IgG3 isotype are effective at recruiting cytotoxic cells that have Fc receptors which will

6   lead to cell killing by monocytes, macrophages, granulocytes and certain lymphocytes.

7   Human antibodies of both the IgG1 and IgG3 isotype mediate ADCC.

8   Another possible mechanism of antibody mediated cancer killing may be through

9   the use of antibodies that function to catalyze the hydrolysis of various chemical bonds in

10   the cell membrane and its associated glycoproteins or glycolipids, so-called catalytic

11   antibodies.

12   There are two additional mechanisms of antibody mediated cancer cell killing

13   which are more widely accepted. The first is the use of antibodies as a vaccine to induce

14   the body to produce an immune response against the putative cancer antigen that resides on

15   the tumor cell. The second is the use of antibodies to target growth receptors and interfere

16   with their function or to down regulate that receptor so that effectively its function is lost.

17   Accordingly, it is an objective of the invention to utilize a method for producing

18   CDMAB from cells derived from a particular individual which are cytotoxic with respect

19   to cancer cells while simultaneously being relatively non-toxic to non-cancerous cells, in

20   order to isolate hybridoma cell lines and the corresponding isolated monoclonal antibodies

21   and antigen binding fragments thereof for which said hybridoma cell lines are encoded.

22   It is an additional objective of the invention to teach CDMAB and antigen binding

1 fragments thereof.

2   It is a further objective of the instant invention to produce CDMAB whose

3 cytotoxicity is mediated through antibody dependent cellular toxicity.

4   It is yet an additional objective of the instant invention to produce CDMAB whose

5 cytotoxicity is mediated through complement dependent cellular toxicity.

6   It is still a further objective of the instant invention to produce CDMAB whose

7 cytotoxicity is a function of their ability to catalyze hydrolysis of cellular chemical bonds.

8   A still further objective of the instant invention is to produce CDMAB, which are

9 useful for in a binding assay for diagnosis, prognosis, and monitoring of cancer.

10   Other objects and advantages of this invention will become apparent from the

11 following description wherein are set forth, by way of illustration and example, certain

12 embodiments of this invention.

13 <u>Brief Description of the Figures:</u>

14 Figure 1: Representative FACS histograms of AR21A51.6, AR26A439.3 and anti-EGFR

15 (positive control) antibody, overlaid onto the isotype negative control antibody, directed

16 against several cancer and non-cancer cell lines.

17

18 <u>Example 1</u>

19 <u>Hybridoma Production – Hybridoma Cell Lines AR21A51.6 and AR26A439.3</u>

20   The hybridoma cell lines AR21A51.6 and AR26A439.3 were deposited, in

21 accordance with the Budapest Treaty, with the American Type Culture Collection, 10801

22 University Blvd., Manassas, VA 20110-2209 on July 1, 2003, under Accession Number

1 PTA-5306 and PTA-5305 respectively. In accordance with 37 CFR 1.808, the depositors

2 assure that all restrictions imposed on the availability to the public of the deposited

3 materials will be irrevocably removed upon the granting of a patent.

4 To produce the hybridoma that produces AR21A51.6 anti-cancer antibody, single

5 cell suspensions of the SW1116 colon cancer cell line that had been grown in SCID mice

6 in order to acquire a solid tumor, were prepared in phosphate buffered saline (PBS).

7 IMMUNEASY™ (Qiagen, Venlo, Netherlands) adjuvant was prepared for use by gentle

8 vortexing. 100 μl of IMMUNEASY™ mouse adjuvant were added to 12 million SW1116

9 cells in the microcentrifuge tube and mixed and left at room temperature for 15 min. 8 to 9

10 week old BALB/c mice were immunized by injecting 50 μl of the antigen-adjuvant

11 containing 2 million cells subcutaneously. Freshly prepared antigen-adjuvant was used to

12 boost the immunized mice 2 and 5 weeks after the initial immunization at 2 million cells in

13 50 μl by a subcutaneous injection. A spleen was used for fusion 3 days after the last

14 immunization. The hybridomas were prepared by fusing the isolated splenocytes with

15 NSO-1 myeloma partners. The supernatants from the fusions were tested for subcloning of

16 the hybridomas.

17 To produce the hybridoma that produces AR26A439.3 anti-cancer antibody, single

18 cell suspensions of frozen patient colon tumor tissue (Genomics Collaborative, Cambridge,

19 MA) were prepared in PBS. IMMUNEASY™ (Qiagen, Venlo, Netherlands) adjuvant was

20 prepared for use by gentle vortexing. 100 μl of IMMUNEASY™ mouse adjuvant were

21 added to 10 million patient tumor cells in the microcentrifuge tube and mixed and left at

22 room temperature for 15 min. 8 to 9 week old BALB/c mice were immunized by injecting

1   50 µl of the antigen-adjuvant containing 2 million cells subcutaneously. Freshly prepared

2   antigen-adjuvant was used to boost the immunized mice 2 weeks after the initial

3   immunization at 2 million cells in 50 µl by a subcutaneous injection. A spleen was used

4   for fusion 3 days after the last immunization. The hybridomas were prepared by fusing the

5   isolated splenocytes with NSO-1 myeloma partners. The supernatants from the fusions

6   were tested for subcloning of the hybridomas.

7   After 1 round of limiting dilution, to determine whether the antibodies secreted by

8   hybridoma cells are of the IgG or IgM isotype, an ELISA assay was employed. 100

9   µl/well of goat anti-mouse IgG + IgM (H+L) at a concentration of 2.4 µg/mL in coating

10   buffer (0.1M carbonate/bicarbonate buffer, pH 9.2-9.6) at 4°C was added to the ELISA

11   plates overnight. The plates were washed thrice in washing buffer (PBS + 0.05 percent

12   Tween). 100 µl/well blocking buffer (5 percent milk in wash buffer) was added to the

13   plate for 1 hr. at room temperature and then washed thrice in washing buffer. 100 µl/well

14   of hybridoma supernatant was added and the plate incubated for 1 hr. at room temperature.

15   The plates were washed thrice with washing buffer and 1/100,000 dilution of either goat

16   anti-mouse IgG or IgM horseradish peroxidase conjugate (diluted in wash buffer with 5

17   percent milkk), 100 µl/well, was added. After incubating the plate for 1 hr. at room

18   temperature the plate was washed thrice with washing buffer. 100 µl/well of TMB

19   solution was incubated for 1-3 minutes at room temperature. The color reaction was

20   terminated by adding 100 µl/well 2M H_2SO_4 and the plate was read at 450 nm with

21   subtraction at 595 nm with a Perkin-Elmer HTS7000 plate reader. As indicated in Table 1

1   the AR21A51.6 and AR26A439.3 hybridoma clones secreted primarily antibodies of the

2   IgG isotype.

3   Hybridoma supernatants were tested for antibodies that bound to target cells in a

4   cell ELISA assay. 2 to 3 colon cancer cell lines were tested: HT-29 and SW1116 (and

5   Lovo for AR26A439.3) and 1 normal cell line: CCD-27sk. The plated cells were fixed

6   prior to use. The plates were washed thrice with PBS containing $MgCl_2$ and $CaCl_2$ at room

7   temperature. 100 μl of 2 percent paraformaldehyde diluted in PBS was added to each well

8   for 10 minutes at room temperature and then discarded. The plates were again washed

9   with PBS containing $MgCl_2$ and $CaCl_2$ 3 times at room temperature. Blocking was done

10   with 100 μl/well of 5 percent milk in wash buffer (PBS + 0.05 percent Tween) for 1 hr at

11   room temperature. The plates were washed thrice with wash buffer and the hybridoma

12   supernatant was added at 100 microliters/well for 1 hr at room temperature. The plates

13   were washed 3 times with wash buffer and 100 μl/well of 1/25,000 dilution of goat anti-

14   mouse IgG or IgM antibody conjugated to horseradish peroxidase (diluted in wash buffer

15   with 5 percent milk) was added. After 1 hr incubation at room temperature the plates were

16   washed 3 times with wash buffer and 100 μl/well of TMB substrate was incubated for 1-3

17   minutes at room temperature. The reaction was terminated with 100 μl/well 2M H_2SO_4 and

18   the plate read at 450 nm with subtraction from 595 nm with a Perkin-Elmer HTS7000 plate

19   reader. The results as tabulated in Table 1 were expressed as the number of folds above

20   background compared to the negative control. The antibody from the AR21A51.6

21   hybridoma had 1.7, 11.4, and 1.3 fold greater binding above background in HT-29,

22   SW1116, and CCD-27sk cells, respectively. This indicated that the antibody bound to an

1 antigen that was expressed more so on some cancer cells versus others and more than on

2 normal skin cells. Conversely, the antibody from the AR26A439.3 hybridoma had 0.7,

3 0.9, 1.5 and 0.8 fold greater binding above background in HT-29, SW1116, Lovo and

4 CCD-27sk cells respectively. According to this assay, the antigen is not being expressed

5 or is expressed at undetectably low levels on these cell lines.

6   In conjunction with testing for antibody binding, the cytotoxic effects of the

7 hybridoma supernatants were tested in the same colon cancer and normal cell lines: HT-29,

8 SW1116 (and Lovo for AR26A439.3) and CCD-27sk. The Live/Dead cytotoxicity assay

9 was obtained from Molecular Probes (Eu, OR). The assays were performed according to

10 the manufacturer's instructions with the changes outlined below. Cells were plated before

11 the assay at the predetermined appropriate density. After 2 days, 100 μl of supernatant

12 from the hybridoma microtitre plates were transferred to the cell plates and incubated in a

13 5 percent CO_2 incubator for 5 days. The wells that served as the positive controls were

14 aspirated until empty and 100 μl of sodium azide (NaN_3) or cycloheximide was added. An

15 isotype control antibody was used that does not bind to HT-29, SW1116, Lovo or CCD-

16 27sk cells and/or a media alone negative control. An anti-EGFR antibody (C225) was also

17 used in the assay for comparison. After 5 days of treatment, the plate was then emptied by

18 inverting and blotting dry. Room temperature DPBS (Dulbecco's phosphate buffered

19 saline) containing $MgCl_2$ and $CaCl_2$ was dispensed into each well from a multichannel

20 squeeze bottle, tapped 3 times, emptied by inversion and then blotted dry. 50 μl of the

21 fluorescent Live/Dead dye diluted in DPBS containing $MgCl_2$ and $CaCl_2$ was added to

22 each well and incubated at 37°C in a 5% CO_2 incubator for 30 minutes. The plates were

read in a Perkin-Elmer HTS7000 fluorescence plate reader and the data was analyzed in
Microsoft Excel. The results are tabulated in Table 1. The AR21A51.6 hybridoma
produced specific cytotoxicity of 11 percent in SW1116 cells, which was 41 percent of the
cytotoxicity obtained with cyclohexamide. The strong binding of AR21A51.6 to SW1116
cells indicated that this level of antibody binding was sufficient to mediate cytotoxicity
against these cancer cells. Although there was weak binding of the AR21A51.6 antibody
to HT-29 colon cancer or CCD-27sk normal skin cells by the cell ELISA assay, this did
not induce cytotoxicity. This suggested that significant antibody binding is required to
mediate cytotoxicity of AR21A51. As tabulated in Table 1, the IgG negative isotype
control did not produce cytotoxicity in the SW1116 cancer cell line. The known non-
specific cytotoxic agents NaN_3 and cycloheximide produced cytotoxicity as expected.

Table 1	Isotype ELISA Fold (above bkgd)		Cytotoxicity (%)								Binding (above bkgd)			
			HT-29		SW1116		Lovo		CCD-27sk		HT-29	SW1116	Lovo	CCD-27sk
	IgG	IgM	Average	CV	Average	CV	Average	CV	Average	CV	Fold	Fold	Fold	Fold
AR21A51.6	66.8	0.9	2	6	11	0			-6	5	1.7	11.4		1.3
AR26A439.3	35.2	3.1	-5	2	-8	4	21	5	-3	4	0.7	0.9	1.5	0.4
Isotype, Media Control			21,25	25,31	-12,-27	-35,-24	-8	-140	1,6	-507,106				
NaN3			59,57	4,9			4	240	14,-4	26,-137				
Cycloheximide			46,48	9,9	27,-2	12,-462	56	11	21,40	21,12				

Results from Table 1 indicate that binding of AR21A51.6 to cancer cells may be an
important step in producing cytotoxicity. The AR26A439.3 hybridoma produced specific
cytotoxicity of 21 percent in Lovo cells, which was 38 percent of the cytotoxicity obtained
with cyclohexamide. There was no detectable or low binding of the AR26A439.3 antibody
to Lovo, HT-29 or SW1116 colon cancer or CCD-27sk normal skin cells by the cell
ELISA assay. This suggested that antibody binding was either occurring at undetectable
levels in this assay or that binding was not necessary to mediate cytotoxicity of
AR26A439.3 against Lovo cells. As tabulated in Table 1, media alone (negative control)

1   did not produce cytotoxicity in the Lovo cancer cell line. The known non-specific

2   cytotoxic agents NaN$_3$ and cycloheximide generally produced cytotoxicity as expected.

3   Example 2

4   Antibody Production:

5   AR21A51.6 and AR26A439.3 monoclonal antibody was produced by culturing the

6   hybridomas in CL-1000 flasks (BD Biosciences, Oakville, ON) with collections and

7   reseeding occurring twice/week and standard antibody purification procedures with Protein

8   G Sepharose 4 Fast Flow (Amersham Biosciences, Baie d'Urfé, QC) were followed. It is

9   within the scope of this invention to utilize monoclonal antibodies that are humanized,

10   chimerized or murine antibodies. AR21A51.6 and AR26A439.3 were compared to a

11   number of both positive (anti-fas (EOS9.1, IgM, kappa, 10 µg/mL, eBioscience, San

12   Diego, CA), anti-Her2/neu (IgG1, kappa, 10 µg/mL, Inter Medico, Markham, ON), anti-

13   EGFR (C225, IgG1, kappa, 5 µg/mL, Cedarlane, Hornby, ON), Cycloheximide (0.5 µM,

14   Sigma, Oakville, ON), and NaN$_3$ (0.1%, Sigma, Oakville, ON)) and negative (107.3 (anti-

15   TNP, IgG1, kappa, 20 µg/mL, BD Biosciences, Oakville, ON), MPC-11 (antigenic

16   specificity unknown, IgG2b, kappa, 20 µg/mL), and IgG Buffer (2%)) controls in a

17   cytotoxicity assay (Table 2). Breast (MDA-MB-231 (MB-231), NCI-MCF-7 (MCF-7)),

18   colon (DLD-1, Lovo, HT-29, SW1116, SW620), ovarian (OVCAR-3 (OVCAR)),

19   pancreatic (BxPC-3), and prostate (PC-3) cancer, and non-cancer skin (CCD-27sk), and

20   lung (Hs888.Lu) cell lines were tested (all from the ATCC, Manassas, VA). The

21   Live/Dead cytotoxicity assay was obtained from Molecular Probes (Eugene, OR). The

1   assays were performed according to the manufacturer's instructions with the changes

2   outlined below. Cells were plated before the assay at the predetermined appropriate

3   density. After 2 days, 100 μl of purified antibody was diluted into media, and then

4   transferred to the cell plates and incubated in a 5 percent CO_2 incubator for 5 days. The

5   plate was then emptied by inverting and blotted dry. Room temperature DPBS containing

6    $MgCl_2$ and $CaCl_2$ was dispensed into each well from a multichannel squeeze bottle, tapped

7   3 times, emptied by inversion and then blotted dry. 50 μl of the fluorescent Live/Dead dye

8   diluted in DPBS containing $MgCl_2$ and $CaCl_2$ was added to each well and incubated at

9    $37^{\circ}C$ in a 5 percent CO_2 incubator for 30 minutes. The plates were read in a Perkin-Elmer

10   HTS7000 fluorescence plate reader and the data was analyzed in Microsoft Excel and the

11   results were tabulated in Table 2. The data represented an average of four experiments

12   tested in triplicate and presented qualitatively in the following fashion: 3/4 to 4/4

13   experiments with >15% cytotoxicity above background (++++), 2/4 experiments with

14   >15% cytotoxicity above background (+++), at least 2/4 experiments with 10-15%

15   cytotoxicity above background (++), and at least 2/4 experiments with 8-10% cytotoxicity

16   above background (+). Unmarked cells in Table 2 represented inconsistent or effects less

17   than the threshold cytotoxicity. The AR21A51.6 antibody produced 130 percent

18   cytotoxicity in the MCF-7 breast cancer cell line relative to the well-described anti-EGFR

19   antibody C225. Further, AR21A51.6 induced significantly higher cytotoxicity against

20   another cancer cell line, compared with C225, the pancreatic cancer cell line BxPC-3.

21   Cytotoxicity on BxPC-3 cells was above that observed with the negative isotype control

22   107.3. The AR26A439.3 antibody produced 36 percent cytotoxicity in the SW1116 colon

cancer cell line relative to C225. In addition, AR26A439.3 triggered cytotoxicity against a variety of other cancer cell lines, compared with C225, the pancreatic cancer cell line BxPC-3, the breast cancer cell line MCF-7 and the prostate cancer cell line PC-3. Cytotoxicity induced by AR26A439.3 on all cancer cell lines was above effects generated by the negative isotype control. Importantly, both AR21A51.6 and AR26A439.3 did not produce cytotoxicity against a number of non-cancer cell lines such as CCD-27sk or Hs888.Lu, indicating that the antibody has specificity towards various cancer cells. The chemical cytotoxic agents induced their expected non-specific cytotoxicity.

Table 2	BREAST		COLON					PANCREAS	OVARY	PROSTATE	NORMAL	
	MB-231	MCF-7	HT-29	DLD-1	Lovo	SW1116	SW620	BxPC-3	OVCAR	PC-3	CCD-27sk	Hs888.Lu
AR21A51.6 (20 µg/mL)		++						++				
AR26A439.3 (20 µg/mL)		++++				++		++		+		
Positive Controls anti-fas (10 µg/mL)		+						++++	++++	++		
anti-Her2/neu (10 µg/mL)												
anti-EGFR (C225, 5 µg/mL)		++		++++	+	++++					++++	
Cycloheximide (0.5 µM)	+++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++
NaN3 (0.1%)	+++	++++	++++	++++	++++	++	++	++++	++++	++++	++	++
Negative Controls 107.3 (IgG1, 20 µg/mL)		++++				+						
MPC-11 (IgG2b, 20 µg/mL)								++				
IgG Buffer (2%)	+											

Cells were prepared for FACS by initially washing the cell monolayer with DPBS (without Ca^{++} and Mg^{++}). Cell dissociation buffer (INVITROGEN, Burlington, ON) was then used to dislodge the cells from their cell culture plates at 37°C. After centrifugation and collection the cells were resuspended in DPBS containing $MgCl_2$, $CaCl_2$ and 2 percent fetal bovine serum at 4°C (staining media) and counted, aliquoted to appropriate cell density, spun down to pellet the cells and resuspended in staining media at 4°C in the presence of test antibodies (AR21A51.6 or AR26A439.3) or control antibodies (isotype control, anti-EGFR, or anti-fas) at 20 µg/mL on ice for 30 minutes. Prior to the addition of

1 Alexa Fluor 488-conjugated secondary antibody the cells were washed once with staining

2 media. The Alexa Fluor 488-conjugated antibody in staining media was then added for 30

3 minutes. The cells were then washed for the final time and resuspended in fixing media

4 (staining media containing 1.5% paraformaldehyde). Flow cytometric acquisition of the

5 cells was assessed by running samples on a FACScan using the CellQuest software (BD

6 Biosciences, Oakville, ON). The forward (FSC) and side scatter (SSC) of the cells were

7 set by adjusting the voltage and amplitude gains on the FSC and SSC detectors. The

8 detectors for the fluorescence (FITC) channel was adjusted by running cells stained only

9 with Alexa Fluor 488-conjugated secondary antibody such that cells had a uniform peak

10 with a median fluorescent intensity of approximately 1-5 units. For each sample,

11 approximately 10,000 stained fixed cells were acquired for analysis and the results

12 presented in Table 3.

13 Table 3 tabulated the mean fluorescence intensity fold increase above isotype

14 control and is presented qualitatively as: between 1.5 to 5 (+); 5 to 25 (++); 25 to 50 (+++);

15 and above 50 (++++). Representative histograms of AR21A51.6 and AR26A439.3

16 antibodies were compiled for Figures 1 and 2 respectively. AR21A51.6 showed high

17 specificity to the colon cancer cell lines DLD-1 and SW1116 with no detectable binding to

18 either normal cell line; CCD-27sk and Hs888.Lu. AR26A439.3 also showed high cancer

19 specificity in that it only bound weakly to the prostate cancer cell line PC-3.

Table 3	BREAST		COLON					PANCREAS	OVARY	PROSTATE	NORMAL	
	MB-231	MCF-7	HT-29	DLD-1	Lovo	SW1116	SW620	BxPC-3	OVCAR	PC-3	CCD-27sk	Hs888.Lu
AR21A51.6 (20 µg/mL)				++++		++						
AR26A439.3 (20 µg/mL)		ND		ND			ND			+		
anti-fas (10 µg/mL)	+	+	+	+	+		+	+	+		+	++
anti-EGFR (C225, 5 µg/mL)	+++	+	+++	+++	++	+		++	++	+++	++	++

20